Virology **283,** 40–48 (2001)
doi:10.1006/viro.2001.0866, available online at http://www.idealibrary.com on IDEAL®

Inhibition of Rb and p53 Is Insufficient for SV40 T-Antigen Transformation

Kris F. Sachsenmeier and James M. Pipas[1]

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received August 14, 2000; returned to author for revision September 13, 2000; accepted February 9, 2001

The SV40 large T-antigen (TAg) has proven useful in studying pathways involved with cell division and tissue homeostasis. TAg disrupts the normal action of tumor suppressors pRb and p53. It is unclear whether T-antigen inhibition of p53 and pRb is sufficient for oncogenic transformation or if additional T-antigen activities are required. To pursue this question, cell lines were generated that coexpress an amino-terminal fragment of T-antigen (TAgN136), which has been shown to be sufficient to block pRb function, together with a dominant-negative p53. Neither focus formation nor saturation density was enhanced by coexpression of the dominant-negative p53 molecule, p53DD, along with TAgN136. Furthermore, a full-length TAg mutant incapable of binding p53 was capable of relieving contact inhibition, a hallmark of transformation. These results suggest the presence of a novel transforming activity in addition to the binding and inactivation of p53, requiring TAg amino acids 137 to 708.

Key Words: T-antigen; dominant-negative p53; transformation; pRb; contact inhibition; saturation density.

INTRODUCTION

Every viral genome is constantly subjected to the ultimate functional genomics screen—that of natural selection. Thus the successful viral proteome represents a unique and powerful set of completed target validation experiments. The study of cellular pathways targeted by viral proteins is validated by the survival and fitness of the viral genome. For example, targets of various tumor virus oncoproteins have proven to be key components of cellular pathways regulating cell division and tissue homeostasis (reviewed by zur Hausen, 1999). Recently, known and unknown targets of the simian virus 40 large T-antigen (TAg) were shown to be among those forming a minimum complement of biochemical pathways the disruption of which is sufficient for oncogenic transformation of normal human cells (Hahn *et al.,* 1999). Viewed from this perspective, the discovery of any novel transforming activity in a viral oncoprotein such as T-antigen has a high probability of revealing some aspect of cellular transformation.

Transforming activities have been mapped to specific regions of the TAg polypeptide by measuring various aspects of the transformed phenotype elicited by wild-type and mutant TAg molecules. Such aspects include immortalization of primary cells (Tevethia *et al.,* 1998), relief from contact inhibition of cellular division at confluence (Price *et al.,* 1994), abnormally high saturation density (Tevethia *et al.,* 1997), the formation of dense foci (Srinivasan *et al.,* 1997), and *in vivo* studies of neoplastic conversion using transgenic mice (reviewed by Stappenbeck *et al.,* 1998).

Amino- and carboxy-terminal transforming activities can act independently, as demonstrated in studies in which coexpression of two separate TAg fragments (amino acids 1 to 147 of TAg and amino acids 251 to 708) resulted in immortalization of primary cells at an efficiency comparable with that of full-length TAg (Tevethia *et al.,* 1998). Amino-terminal activities include the binding and inactivation of tumor suppressor pRb (reviewed by Livingstone, 1992) as well as a DnaJ chaperone-like activity (reviewed by Brodsky and Pipas, 1998). A fragment of TAg comprising only amino acids 251 to 708 was shown to cooperate with an activated *ras* oncogene to form dense foci in primary rat embryo fibroblasts (Cavender *et al.,* 1995). An essential role for carboxy-terminal regions of TAg during transformation was demonstrated in studies using transgenic mice expressing wild-type and mutant TAgs in the small intestine (Kim *et al.,* 1994). Whereas full-length wild-type TAg induced intestinal dysplasia, expression of amino-terminal fragments resulted in only hyperplasia (Kim *et al.,* 1994). Furthermore, amino-terminal fragments of SV40 TAg have been shown to be either impaired or completely deficient for transformation in dense focus assays using either C3H 10T1/2 cells or REF52 cells, respectively (Srinivasan *et al.,* 1997). Carboxy-terminal regions of TAg responsible for binding and inactivating tumor suppressor p53 function (Kierstead and Tevethia, 1993) are among those absent from amino-terminal fragments in studies cited above. These findings suggest an important role for p53 in tumor suppression. However, they also raise the question of whether p53 is the only cellular target of full-length TAg left unaltered by amino-terminal TAg fragments.

In this study, we examined the hypothesis that cellular

[1] To whom correspondence should be addressed. Fax: (412) 624-4759. E-mail: pipas@pitt.edu.

0042-6822/01 $35.00

p53 is the sole target for the carboxy terminus of TAg during transformation by using cells expressing both dominant-negative mutant p53DD and TAg amino-terminal fragment TAgN136. As a truncation mutant comprising only the wild-type p53 oligomerization domain, p53DD disrupts normal p53 transcriptional transactivation functions by forming inactive heterooligomers with endogenous full-length p53 (Shaulian *et al.,* 1992, 1995). If p53 is the sole target of TAg amino acids 137 to 708, then transformation in the presence of p53DD should be as efficient for TAg p53 binding mutants as for wild-type TAg.

RESULTS

The T-antigen LXCXE motif is not sufficient for high saturation density

In order to further investigate defects in transformation by amino-terminal TAg fragments, the saturation densities of stable cell lines expressing TAg (10T TAg) and TAg mutants (N136 and N136-3213) were studied. Mutant N136 comprises the first 136 amino acids of wild-type T-antigen (Srinivasan *et al.,* 1997). N136-3213 is identical to N136 except that it encodes two point mutations (E107K, E108K) in the TAg LXCXE motif required for binding and inactivation of tumor suppressor pRb (DeCaprio *et al.,* 1988). C3H10T1/2 cells were transfected and selected for antibiotic resistance, and expression of the transfected genes was verified by immunoblotting and immunofluorescence (Fig. 3 and data not shown). Two days after reaching confluence, cells were fixed and stained with DNA-specific dye Hoechst 33258. As seen in Fig. 1, the saturation density of 10T TAg cells was dramatically higher than that of control cells transfected with empty vector alone (10T). In at least 10 independent experiments, cells expressing wild-type TAg, reached a minimum twofold increase over normal, contact-inhibited saturation densities of approximately 5×10^4 cells per square centimeter. A complete loss of contact inhibition in 10T TAg cells was indicated by the presence of metaphase mitotic figures (see arrowhead in Fig. 1). After approximately 3 days of postconfluent culture, cells expressing full-length TAg supersaturated the culture vessel, as indicated by a precipitous drop in culture medium pH (data not shown).

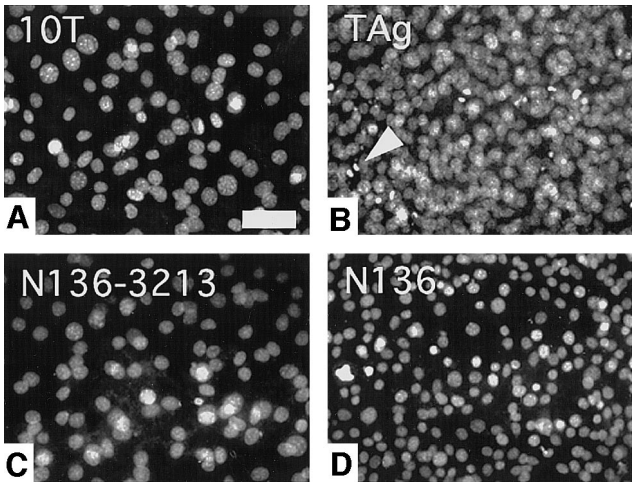

FIG. 1. The T-antigen LXCXE motif is insufficient for high saturation density. C3H10T1/2 cells expressing a neomycin resistance gene (A), wild-type SV40 T-antigen (B), TAgN136-3213 (C), and TAgN136 (D) were fixed and stained with DNA-specific dye Hoechst 33258 two days after reaching confluence. Microphotographs of stained nuclei were made using UV ilumination. The arrowhead in B indicates a mitotic figure. Bar, 10 μm.

Typical of cells experiencing a pRb-dependent G1/S cell-cycle block (Liu and Preisig, 1999; Rao, 1999), nuclei in contact-inhibited cells expressing no TAg (10T) or TAgN136-3213 were larger than nuclei of cells expressing an intact TAg LXCXE pRb-binding motif and J-domain (Fig. 1). The relatively normal saturation density of TAgN136 cells indicates a requirement for full-length TAg in achieving the high saturation density characteristic of transformed cells.

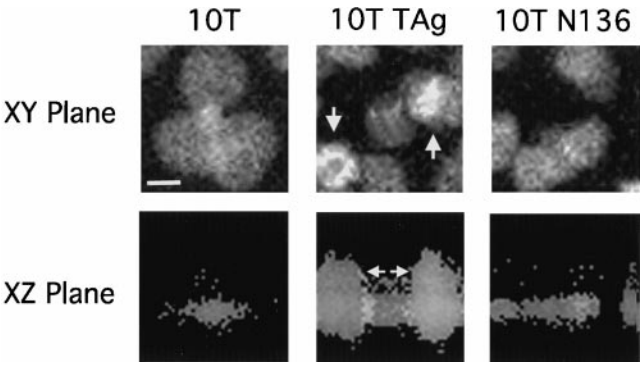

FIG. 2. Stacking of cells expressing full-length SV40 T-antigen. Two days after reaching confluence, C3H10T1/2 cells expressing wild-type TAg and T-antigen mutant N136 were fixed, treated with RNase, and stained with propidium iodide. Stained nuclei were visualized in two planes by laser scanning confocal microscopy. Arrows indicate two nuclei above the plane of the confluent monolayer. Bar, 1 μm.

Full-length T-antigen promotes three-dimensional growth above a confluent monolayer

High saturation density (see Fig. 1) could arise either from increased packing of cells in a single layer or from an increased capacity for detachment from the culture substratum and growth above the monolayer. The nature of the high saturation phenomenon was studied using three-dimensional confocal microscopic images taken from saturated cultures of cells expressing full-length TAg and TAgN136. Figure 2 displays optical slices taken in the X-Y plane of saturated cultures of cells. This view offers a cross-sectional or "top-to-bottom" picture of the relative position of nuclei in cells at postconfluent density. Two days after reaching confluence, cells were

stained with nucleic acid-specific dye propidium iodide following fixation and RNase treatment. Nuclei in a plane above that of the confluent monolayer were visible only in cells expressing full-length T-antigen compared with 10TN136 or control C3H10T1/2 cells (Fig. 2). These results demonstrate a role for TAg amino acids 137 to 708 in promoting growth above that of the saturated monolayer characteristic of contact-inhibited cells.

p53DD fails to enhance saturation density of cells expressing p53-binding mutants of T-antigen

Regions in the carboxyl half of the TAg polypeptide required to bind and inactivate tumor suppressor p53 are not present in amino-terminal fragment N136. We hypothesized that if p53 was the sole target of TAg amino acids 137–708, then the failure of 10TN136 cells to reach high saturation density would be complemented by a dominant-negative p53 mutant. Stable C3H10T1/2 cell lines were made expressing full-length TAg, fragment N136, as well as p53DD alone or in combination with TAgN136. Lines were also made expressing a full-length p53-binding mutant TAg, 5031. TAg mutant 5031 contains three point mutations (D402N, V404N, V413M) in the TAg p53-binding region that abolish p53 binding and inactivation (Peden *et al.,* 1998). Expression of wild-type p53 and p53DD was confirmed by immunofluorescence and immunoblotting as shown in Fig. 3. The inhibitory activity of mutant p53DD upon endogenous wild-type p53 was indicated by a dramatic reduction in steady state levels of the p53-dependent protein p21 (Fig. 3). TAg and mutant TAg expression in the same cells and lysates was also confirmed and expression was shown by immunofluorescence for at least 70% of the cells for each line (data not shown). As expected, full-length p53 was only detectable in cells in which its normally rapid turnover was stabilized by wild-type TAg (Fig. 3).

The ability of p53DD to overcome contact inhibition and increase the saturation density of 10TN136 cells was measured by trypsinizing and counting the number of cells present for each cell line 2 days after reaching confluence. As shown in Fig. 4, p53DD achieved only a modest increase in 10TN136 saturation density. Furthermore, full-length TAg mutant 5031 was able to overcome contact inhibition, resulting in a saturation density comparable with that of wild-type TAg, despite its inability to bind or stabilize p53.

p53DD promotes attachment but not survival of cells expressing TAg fragment N136

Cell death and/or detachment to the culture medium might account for the low saturation density of 10TN136 cells. Detached or dead cells would not be detectable in the assays described above. Therefore, cell death and detachment phenomena were studied by counting the total number of cells (i.e., attached and detached) as

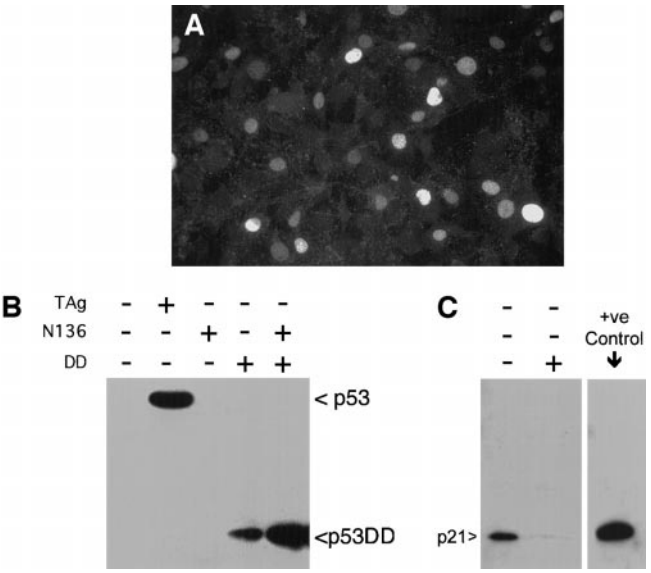

FIG. 3. Expression of p53DD in C3H10T1/2 cells. C3H10T1/2 cells were stably transfected with genomic clones of a p53DD, TAg, TAg amino-terminal fragment N136 alone, or p53DD and empty vector DNA as a control. Following G418 selection, cells were screened for expression of the transfected oncoproteins. For immunofluorescence (A), cells were cultured on coverslips, fixed, and incubated with anti-p53 monoclonal antibody 421. For immunoblotting (B), equal amounts of TX100 soluble protein were resolved on a 12% SDS–PAGE gel, transferred to a membrane, and probed with monoclonal antibody 421. Similar immunoblots, including a positive control of purified p21, were probed with anti-p21 antibody in C.

cells reached and passed confluence and by measuring their colony-forming efficiency.

Two days after reaching confluence, C3H10T1/2 cells expressing either wild-type TAg, N136, 5031, and p53DD alone or in combination were trypsinized and counted. Culture medium was not changed during the 2-day period but was collected and concentrated and the fraction of detached cells was calculated as a percentage of the total number attached and detached. Data presented in Fig. 5 show a dramatic difference in the fraction of cells detached between 10TN136 cells and all other cells examined. Similar detachment was also seen in BALB/c 3T3 cells expressing the identical oncoproteins (data not shown). This difference increased linearly until cells expressing full-length TAg supersaturated the culture vessel.

Interestingly, detachment of cells expressing both TAgN136 and p53DD was strikingly reduced compared with that of cells expressing TAgN136 alone. p53DD reduced the fraction of detached 10TN136 cells approximately fivefold (Fig. 5), suggesting a need for p53 inactivation in promoting cell attachment during transformation by T-antigen.

To examine the potential contribution of p53-dependent apoptosis to the low saturation density and high rate of postconfluent detachment, the colony-forming ef-

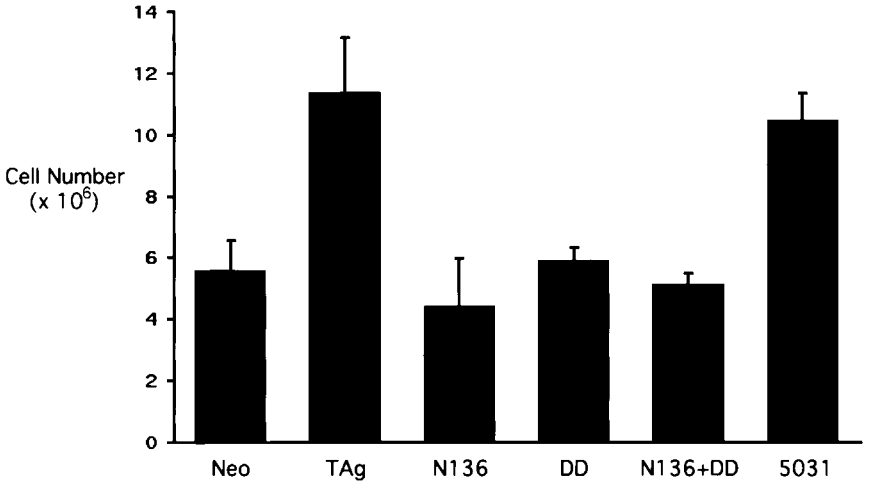

FIG. 4. p53DD fails to enhance saturation density of cells expressing p53-binding mutants of T-antigen. C3H10T1/2 cells stably expressing the indicated oncoproteins or a neomycin resistance gene alone were cultured 2 days past confluence. Cells were trypsinized and counted using a hemacytometer. Error bars represent the standard error of three independent experiments.

ficiency of cells expressing TAgN136 and p53DD was measured and compared to that of control cells. Cells expressing TAgN136 showed only a slight difference in colony-forming efficiency compared with those expressing p53DD alone or TAgN136 in addition to p53DD (Fig. 6). Cells expressing full-length TAg were approximately twice as efficient at colony formation as the other lines tested, with the exception of 10T5031, which showed an intermediate survival rate (Fig. 6). Similar results were obtained using cells that had spontaneously detached during postconfluent culture (data not shown). These results indicate that the presence of an apoptotic subpopulation unique to cells expressing TAgN136, either adherent or detached, cannot be detected by a difference in colony-forming efficiency.

p53DD fails to enhance focus formation by TAg amino-terminal fragment N136 and full-length TAg mutant TAg5031

In order to confirm the failure of p53DD to enhance transformation by TAg p53-binding mutants, a different assay of *in vitro* transformation was used. Dense focus formation assays were performed using REF52 cells, in

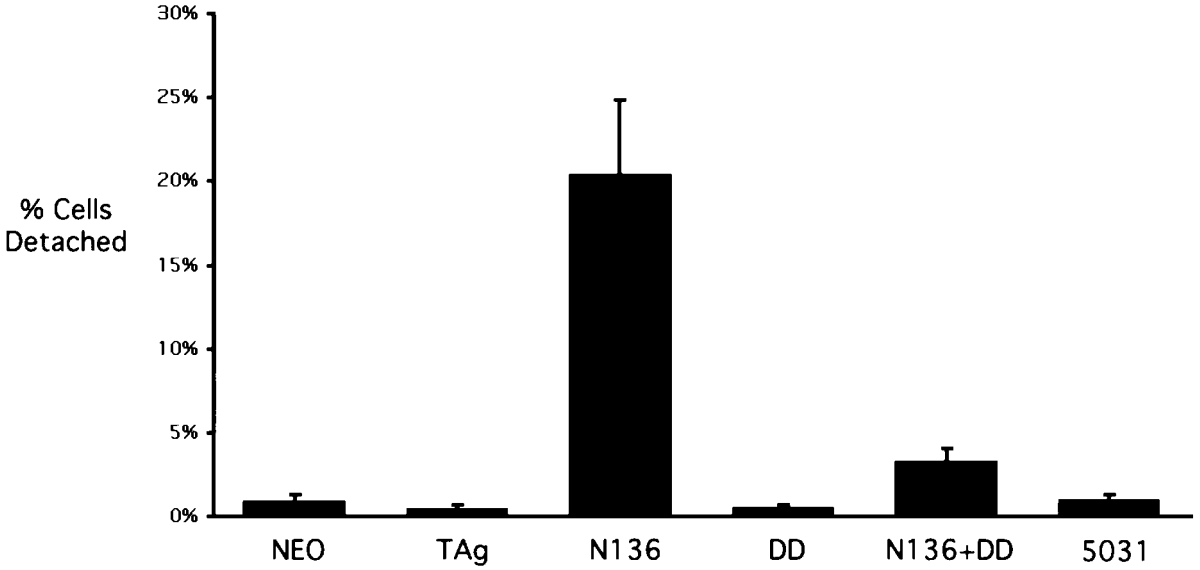

FIG. 5. p53DD promotes the attachment of cells expressing TAg mutant N136. C3H10T1/2 cells stably expressing the indicated oncoproteins or a neomycin resistance gene were cultured two days past confluence. Attached cells were trypsinized and counted using a hemacytometer. Cells found in the culture medium were concentrated and counted. The percentage of cells detached was calculated as [detached/(detached + attached)] × 100. Error bars represent the standard error of three independent experiments.

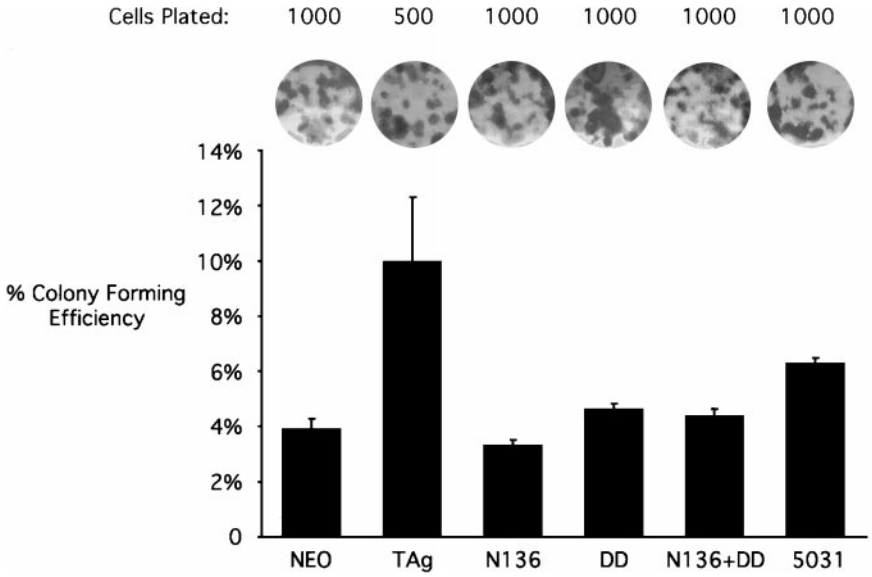

FIG. 6. p53DD fails to promote survival of cells expressing TAg mutant N136. Two days after reaching confluence, C3H10T1/2 cells stably expressing the indicated oncoproteins or a neomycin resistance gene were trypsinized and counted. An equal number of cells from each line was replated at various dilutions and cultured for 8 to 10 days. The resulting colonies were fixed, stained, and counted, and the fraction of surviving cells capable of forming colonies was expressed as a percentage of the cells plated. Stained colonies from each cell line are shown, although not necessarily at the plating density optimal for colony counting. Data are representative of at least three independent experiments. Error bars represent the standard error of triplicate plates.

which TAgN136 and TAg5031 are incapable of focus formation (Srinivasan *et al.,* 1997). As a less stringent test of focus formation, C2H10T1/2 cells, which show only a 10% focus-forming efficiency compared with that of wild-type TAg (Srinivasan *et al.,* 1997), were used. Cells were transfected with equal amounts of plasmid containing genomic clones of full-length TAg, TAgN136, TAg5031, p53DD, or a combinations of p53DD with either TAgN136 or TAg5031. Following transfection, cells were split into triplicate vessels for a focus assay as described under Materials and Methods. As shown in Fig. 7, p53DD neither complemented the deficiency of TAgN136 for focus formation in REF52 cells nor enhanced the ability of TAgN136 or TAg5031 to induce foci at wild-type TAg efficiency in C3H10T1/2 cells. Transfection of empty neomycin vector plasmid DNA failed to yield foci in either

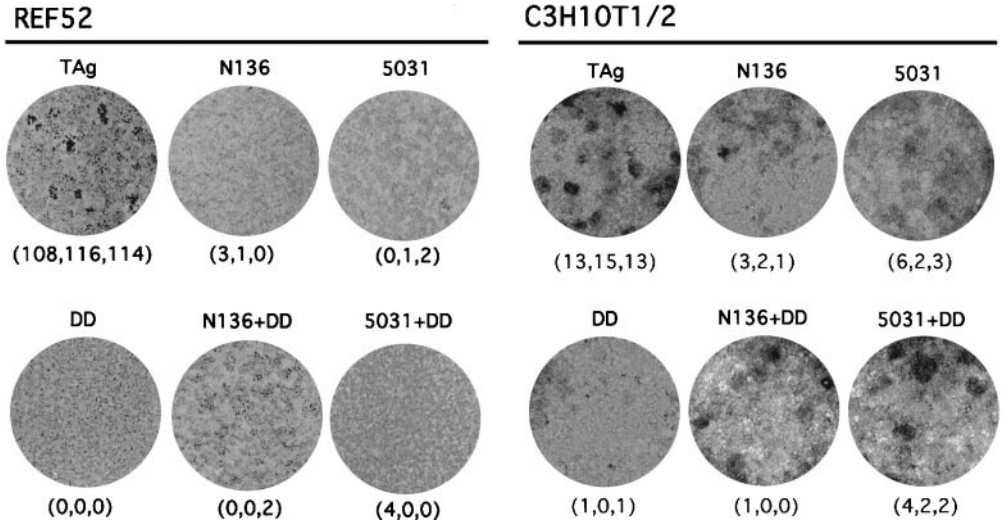

FIG. 7. p53DD fails to enhance focus formation by TAg amino-terminal fragment N136. REF52 and C3H10T1/2 cells were transfected with plasmids containing genomic clones of wild-type TAg, TAgN136, TAg5031, p53DD, and combinations of p53DD with either TAgN136 or 5031. Two days following transfection, cells were split onto triplicate plates and cultured for 6 (REF52 in 75-cm^2 flasks) or 3 (C3H10T1/2 in 3.5-cm diameter plates) weeks. Cells were then fixed and stained and dense foci were counted. Examples of dense foci are shown without magnification for comparison of focus morphology. Numbers in parentheses are counts from triplicate wells and are representative of at least three independent experiments.

cell line, and stable expression of the transfected proteins was confirmed by immunoblotting and immunofluorescence (Fig. 3 and data not shown).

DISCUSSION

Experiments reported here suggest the presence of a novel carboxy-terminal transforming activity of TAg, in addition to the binding and inactivation of p53. They preclude an essential role for cellular p53 as the sole cellular target of the carboxyl half of SV40 large T-antigen during transformation *in vitro.* Despite the presence of intact pRb-binding and chaperone-like DnaJ domains, amino-terminal TAg fragment N136 failed to induce high saturation density, consistent with its previously published (Srinivasan *et al.,* 1997) deficiency in dense focus formation. This failure is demonstrated to arise, in part, from the detachment of cells expressing TAgN136 from the culture dish after overcoming contact inhibition. Dominant-negative mutant p53DD was capable of reducing cellular detachment when coexpressed with TAgN136 but not capable of inducing saturation densities comparable with those achieved by either wild-type TAg or a full-length p53-binding mutant of TAg, TAg5031. Furthermore, the possible contribution of p53-dependent apoptosis in reducing the transformation efficiency of TAgN136 was ruled out by experiments showing comparable colony-forming efficiencies between cells expressing TAgN136 and TAgN136 along with p53DD. Finally, a combination of p53 and TAgN136 or TAg5031 was not able to enhance the ability of these mutants to form dense foci.

Two possible mechanisms that could account for the apparent low saturation density (Fig. 2) and reduced focus-forming ability of TAgN136 (Srinivasan *et al.,* 1997) were studied: contact inhibition and TAgN136-induced cell death. Simple contact inhibition in cells expressing TAg amino-terminal fragment N136 is curious given that it contains the two amino-terminal activities shown (Zalvide *et al.,* 1998) to be essential in activation of pRb family proteins: an intact "J domain" as well as the LXCXE motif. Both of these activities have been shown to be required in *cis* for transformation *in vitro* (Srinivasan *et al.,* 1997; Slinskey *et al.,* 1999). For example, 10TN136 cells might bypass pRb-mediated contact inhibition, continue to divide after reaching confluence, and simply detach into the culture medium due to the absence of some novel proadhesive activity present in full-length TAg. Such an activity is suggested by both the high frequency of detached 10TN136 cells as well as the ability of full-length TAg to promote three-dimensional growth. However, only approximately 20% of the total number of 10TN136 cells was found floating in the culture medium. Thus, simple detachment following LXCXE-mediated release from pRb-mediated contact inhibition cannot account for the 250% difference in saturation density observed between TAgN136 and full-length TAg (Fig. 3).

A role for TAgN136-mediated induction of apoptosis was eliminated as a potential cause for the low transformation efficiency of TAgN136. Analogous induction of apoptosis has been reported for adenovirus oncoprotein E1A which, like TAgN136, possesses an LXCXE motif (Dyson *et al.,* 1989). Inactivation of pRb by E1A is believed to activate the transcription of E2F responsive gene p19ARF and subsequent p19ARF-mediated inactivation of p53 inhibitor MDM2 (Lowe *et al.,* 1993). The resultant stabilization of p53 was shown to induce p53-dependent apoptosis in rodent cells (Lowe *et al.,* 1993).

Analysis of cell death in a system in which cells detach spontaneously is complicated by the potential induction of apoptosis due to loss of adhesion alone (Sachsenmeier *et al.,* 1996; reviewed by Ruoslahti and Reed, 1994). Detached cells might undergo apoptosis simply from TAgN136-induced detachment, independently of p53 stabilization. Nevertheless, the possibility of p53-induced apoptosis in 10TN136 cells was examined by replating both attached and detached cells after reaching confluence and measuring colony-forming efficiency. If the TAgN136 was responsible for the induction of cell death, either before or immediately after detachment, then a TAgN136-specific decrease in cell survival should become evident. Apoptosis due to p53-induction should be counteracted by p53DD. As shown in Fig. 6, the colony-forming efficiency of 10TN136 cells was not appreciably different from that of control cells or cells expressing p53DD and TAgN136. An increase in steady state levels of wild-type p53 was also absent in adherent cells expressing TAgN136 (Fig. 3).

Data reported here using p53 binding mutants suggest a contributing role for p53 as a cellular target during transformation by TAg. The ability of p53DD to abrogate detachment of 10TN136 cells (Fig. 5) is interesting given reports of p53-dependent apoptosis following integrin-specific loss of cell-substrate adhesion (Ilic *et al.,* 1998). Furthermore, the modest difference (approximately 20%) in mean saturation density seen between 10TN136 cells with and without p53DD (Fig. 3) matches the 20% fraction of 10TN136 cells detached. These data are supportive of a model whereby a fraction of contact-uninhibited cells are stimulated to undergo p53-dependent apoptosis during an abortive attempt to detach from the culture plate and grow above the monolayer, as seen with full-length TAg in Figs. 2 and 3. In fact, the failure of full-length p53-binding mutant TAg5031 to achieve wild-type TAg colony-forming efficiency (Fig. 6) suggests a role, albeit insufficient, for p53 as a contributing cellular target during TAg transformation. That is, although failure of TAg5031 to inactivate p53 may contribute to an intermediate survival rate in replated cells (Fig. 6), untargeted cellular p53 did not disrupt the ability of TAg5031 to both promote saturation density (Fig. 4) and suppress detach-

ment (Fig. 5). Taken together, these data do not support a primary role for p53-mediated apoptosis in reducing the saturation density of cells expressing TAgN136.

These results raise at least two crucial questions regarding pathways leading to cellular transformation. First, these studies suggest that TAg action upon cellular p53 is not equivalent to inhibition of p53 activity as achieved solely by p53DD (Shaulian *et al.,* 1995) alone. Thus, how do p53 and p53-dependent pathways in cells expressing p53DD differ from those in the presence of full-length TAg? This question is intriguing given the capabilities of both TAg (Rushton *et al.,* 1997; Quartin *et al.,* 1994) and p53DD (Shaulian *et al.,* 1995) to disrupt both cell-cycle inhibition and transcriptional activation by endogenous p53.

The second question raised by these experiments is whether a novel transforming activity requiring the carboxy half of TAg is responsible for wild-type TAg transformation. The failure of p53DD to complement the saturation density (Figs. 1 and 3) and focus-formation (Fig. 7) deficiencies of TAgN136 and Tag5031 suggests at least two possibilities: (1) Some other activity of wild-type TAg, other than p53-inactivation and deficient in TAgN136, is required for transformation. One such candidate activity is that of the endogenous ATPase of TAg that maps to the carboxyl terminus of TAg (Clark *et al.,* 1983). The importance of the ATPase activity of TAg for transformation is questionable, however, because ATPase mutant TAg5061 transforms C3H10T1/2 cells and REF52 cells with wild-type efficiency (Peden *et al.,* 1998). (2) The inactivation of cellular p53 by wild-type TAg is not equivalent to that of p53DD. In this case, p53DD could not make the same contribution to *in vitro* transformation made by a full-length wild-type TAg molecule, missing in TAgN136. Whereas these experiments do not rule out a need for p53 inactivation along with the putative novel transforming activity, they strongly suggest a requirement of this activity in addition to the p53-binding, pRB-binding, and J domain-dependent activities already shown to be present in TAg.

The high saturation density achieved by full-length p53-binding mutant TAg5031 (Fig. 3) suggests a fundamental difference between saturation density and dense focus formation as measures of *in vitro* transformation. Similar to TAgN136, TAg5031 has been shown to be less efficient than wild-type TAg at dense focus formation (Srinivasan *et al.,* 1997). Growth above the confluent monolayer was observed for cells expressing TAg5031 (data not shown) and this ability is likely responsible for the near wild-type saturation density observed for TAg5031 (Fig. 3). Further study is needed to determine why TAg5031 focus-forming efficiency should differ from its ability to reach high saturation. It is important to note that in these experiments, saturation density was measured using stable transfected cells while dense focus assays are performed without selection for drug resistance.

Finally, these experiments introduce an assay system to screen candidate molecules for involvement in novel transformation pathways. REF52 cells stably expressing both p53DD and TAgN136 are unique in that all of the best characterized cellular targets required for disruption during oncogenic transformation by TAg (tumor-suppressors pRb, p53, and J-domain-susceptible targets) are presumably acted upon by either TAgN136 or p53DD. While phenotypically normal with regard to dense focus formation, yet highly abnormal for cell-growth control and apoptotic potential, REF cells expressing TAgN136 and p53DD offer a powerful and sensitive measure of potentially unknown or subtle transforming molecules and cellular effectors.

MATERIALS AND METHODS

Cells

C3H10T1/2 cells (ATCC No. CCL-226) and REF52 cells are immortalized embryo fibroblastic lines derived from mouse and rat, respectively. These lines were chosen for their low rate of spontaneous transformation. Cells were cultured in modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2. Cells were passaged before reaching confluence except as indicated.

Plasmids

Expression of genomic clones of wild-type SV40 T-antigen, T-antigen-amino terminal fragment N136 and p53-binding mutant 5031 were driven by either the Rous sarcoma virus promoter or the SV40 promoters, and the constructs have been previously described (Srinivasan *et al.,* 1997). Each of the constructs express small t-antigen. Expression of p53DD was driven by the cytomegalovirus promoter in plasmid pCMVp53DD and was a kind gift of Dr. Moshe Oren (The Weizmann Institute, Rehovot, Israel). All plasmids contained either a neomycin/geneticin or hygromycin resistance gene.

Dense focus assays

Dense focus assays were performed as described (Srinivasan *et al.,* 1997). Before reaching confluence, 1 μg (C3H10T1/2) or 2 μg (REF52) of circular plasmid DNA was transfected into cells using lipofectamine (Life Technologies) according to the manufacturer's instructions. Following transfection, cells were split into triplicate dishes. One-third of the transfected C3H10T1/2 cells were split into each of three wells of a six-well culture dish. All of the transfected REF52 cells were split into three different 75-cm^2 culture flasks. Cells were cultured

with twice-weekly feedings for 3 (C3H10T1/2) or 6 weeks (REF52) until foci were evident. At this time, cells were rinsed, fixed with 10% formalin and stained with methylene blue, and the number of foci in each well was counted.

Transfection

Transfection of plasmid DNA was performed essentially as described (Srinivasan *et al.,* 1997) and in parallel with dense focus assays. A total of 1 to 2 μg of DNA was transfected as described for dense focus assays and the transfected cells were selected for geneticin (G418) or hygromycin resistance at concentrations of 400 and 200 μg per milliliter of selective medium, respectively.

Immunofluorescence

Cells cultured on glass coverslips were rinsed twice with phosphate-buffered saline (PBS) at room temperature, fixed with ice-cold methanol, and frozen at −20°C. After thawing, cells were permeabilized for 5 min at 37°C using 0.1% Triton X-100 in PBS and incubated for 1 h at 37°C with primary antibody at a dilution of 1:300 in PBS. Primary antibodies included monoclonal antibody 419 for SV40 TAg and mutants and monoclonal antibody 421 for p53 and p53DD. Two rinses in PBS were followed by 30-min incubation with fluorescein-conjugated goat anti-mouse secondary antibody (Sigma Chemical Co., St. Louis, MO) at 37°C. Next, cells were rinsed twice with PBS, counterstained for 5 min at 37°C with Hoechst 33258 (Roche Molecular Biochemicals, Indianapolis, IN) at 5 μg per milliliter in PBS, and again rinsed twice with PBS. Finally, cells were mounted using Vecta Shield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and viewed under UV and fluorescent light. Microphotographs were taken using a Nikon Axiophot microscope.

Immunoblotting

Cells were grown to the indicated densities, rinsed twice with ice-cold PBS, and collected by scraping the plates on ice in Triton lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM sodium fluoride, 100 mM sodium orthovanadate, Roche Complete protease inhibitor cocktail, 1 μM pepstatin). Lysates were clarified by centrifugation at 14,000*g* and frozen at −80°C. Protein concentrations were determined by the bichinoic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. Immunoblotting was performed as described (Srinivasan *et al.,* 1997) with minor modifications. Briefly, equal portions of protein were resolved on a 12% polyacrylamide gel containing sodium dodecyl sulfate, transferred to a polyvinyl difluoride membrane (Millipore, Bedford, MA), and probed using monoclonal antibodies to TAg (Mab419) or p53 (MAb421). Specific protein bands were detected using horseradish peroxidase-conjugated goat anti-mouse secondary antibody and chemiluminescent substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

Confocal microscopy

Postconfluent cells were rinsed twice with PBS, fixed with ice-cold methanol, and treated with 100 μg/ml RNase A (Roche) for 10 min at 37°C to remove RNA. Next, cells were stained with nucleic acid intercalating dye prodidium iodide (Sigma) at 50 μg/ml in PBS. Cells were then rinsed once in PBS and mounted using Vecta Shield mounting medium (Vector Laboratories). A z-series of 250-nm optical slices was taken using a Ziess LSM210 confocal microscope. Digital images were processed for three-dimensional visualization using the NIH Image software package.

ACKNOWLEDGMENTS

The authors thank members of the Pipas Laboratory and Dr. Jeffrey Brodsky for constructive criticism of the manuscript. We also thank Patrick Carroll for technical assistance. This work was supported by Grant CA40586 from the National Institutes of Health to J.M.P. and Grant CA76733 to K.F.S.

REFERENCES

Brodsky, J. L., and Pipas, J. M. (1998). Polyomavirus T antigens: Molecular chaperones for multiprotein complexes. *J. Virol.* **72**(7), 5329–5334.

Cavender, J. F., Conn, A., Epler, M., Lacko, H., and Tevethia, M. J. (1995). Simian virus 40 large T antigen contains two independent activities that cooperate with a ras oncogene to transform rat embryo fibroblasts. *J. Virol.* **69**(2), 923–934.

Clark, R., Peden, K., Pipas, J. M., Nathans, D., and Tjian, R. (1983). Biochemical activities of T-antigen proteins encoded by simian virus 40 A gene deletion mutants. *Mol. Cell. Biol.* **3,** 220–228.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54,** 275–283.

Dyson, N., Buchkovich, K., Whyte, P., and Harlow, E. (1989). The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. *Cell* **58,** 249–255.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements [see comments]. *Nature* **400,** 464–468.

Ilic, D., Almeida, E. A., Schlaepfer, D. D., Dazin, P., Aizawa, S., and Damsky, C. H. (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J. Cell Biol.* **143,** 547–560.

Kierstead, T. D., and Tevethia, M. J. (1993). Association of p53 binding and immortalization of primary C57BL/6 mouse embryo fibroblasts by using simian virus 40 T-antigen mutants bearing internal overlapping deletion mutations. *J. Virol.* **67**(4), 1817–1829.

Kim, S. H., Roth, K. A., Coopersmith, C. M., Pipas, J. M., and Gordon, J. I. (1994). Expression of wild-type and mutant simian virus 40 large tumor antigens in villus-associated enterocytes of transgenic mice. *Proc. Natl. Acad. Sci. USA* **91,** 6914–6918.

Liu, B., and Preisig, P. (1999). TGF-beta1-mediated hypertrophy involves

inhibiting pRB phosphorylation by blocking activation of cyclin E kinase. *Am. J. Physiol.* **277**(2 Pt 2), F186–F194.

Livingston, D. M. (1992). Functional analysis of the retinoblastoma gene product and of RB-SV40 T antigen complexes. *Cancer Surv.* **12,** 153–160.

Lowe, S. W. and Ruley, H. E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 EIA and accompanies apoptosis. *Genes Dev.* **7**(4), 535–545.

Peden, K. W., Srinivasan, A., Vartikar, J. V., and Pipas, J. M. (1998). Effects of mutations within the SV40 large T antigen ATPase/p53 binding domain on viral replication and transformation. *Virus Genes* **16,** 153–165.

Pipas, J. M. (1998). Molecular chaperone function of the SV40 large T antigen. *Dev. Biol. Stand.* **94,** 313–319.

Price, T. N., Moorwood, K., James, M. R., Burke, J. F., and Mayne, L. V. (1994). Cell cycle progression, morphology and contact inhibition are regulated by the amount of SV40 T antigen in immortal human cells. *Oncogene* **9,** 2897–2904.

Quartin, R. S., Cole, C. N., Pipas, J. M., and Levine, A. J. (1994). The amino-terminal functions of the simian virus 40 large T antigen are required to overcome wild-type p53-mediated growth arrest of cells. *J. Virol.* **68**(3), 1334–1341.

Rao, G. N. (1999). Differential regulation of p27kip1 levels and CDK activities by hypertrophic and hyperplastic agents in vascular smooth muscle cells. *Biochim. Biophys. Acta* **1448,** 525–532.

Ruoslahti, E., and Reed, J. C. (1994). Anchorage dependence, integrins, and apoptosis. *Cell* **77,** 477–478.

Rushton, J. J., Jiang, D., Srinivasan, A., Pipas, J. M., and Robbins, P. D. (1997). Simian virus 40 T antigen can regulate p53-mediated transcription independent of binding p53. *J. Virol.* **71**(7), 5620–5623.

Sachsenmeier, K. F., Sheibani, N., Schlosser, S. J., and Allen-Hoffmann, B. L. (1996). Transforming growth factor-beta 1 inhibits nucleosomal fragmentation in human keratinocytes following loss of adhesion. *J. Biol. Chem.* **271,** 5–8.

Shaulian, E., Haviv, I., Shaul, Y., and Oren, M. (1995). Transcriptional repression by the C-terminal domain of p53. *Oncogene* **10**(4), 671–680.

Shaulian, E., Zauberman, A., Ginsberg, D., and Oren, M. (1992). Identification of a minimal transforming domain of p53: Negative dominance through abrogation of sequence-specific DNA binding. *Mol. Cell. Biol.* **12,** 5581–5592.

Slinskey, A., Barnes, D., and Pipas, J. M. (1999). Simian virus 40 large T antigen J domain and Rb-binding motif are sufficient to block apoptosis induced by growth factor withdrawal in a neural stem cell line. *J. Virol.* **73,** 6791–6799.

Srinivasan, A., McClellan, A. J., Vartikar, J., Marks, I., Cantalupo, P., Li, Y., Whyte, P., Rundell, K., Brodsky, J. L., and Pipas, J. M. (1997). The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Mol. Cell. Biol.* **17,** 4761–4773.

Stappenbeck, T. S., Wong, M. H., Saam, J. R., Mysorekar, I. U., and Gordon, J. I. (1998). Notes from some crypt watchers: Regulation of renewal in the mouse intestinal epithelium. *Curr. Opin. Cell Biol.* **10,** 702–709.

Tevethia, M. J., Lacko, H. A., Kierstead, T. D., and Thompson, D. L. (1997). Adding an Rb-binding site to an N-terminally truncated simian virus 40 T antigen restores growth to high cell density, and the T common region in trans provides anchorage-independent growth and rapid growth in low serum concentrations. *J. Virol.* **71**(3), 1888–1896.

Tevethia, M. J., Lacko, H. A., and Conn, A. (1998). Two regions of simian virus 40 large T-antigen independently extend the life span of primary C57BL/6 mouse embryo fibroblasts and cooperate in immortalization. *Virology* **243,** 303–312.

Zalvide, J., Stubdal, H., and DeCaprio, J. A. (1998). The J domain of simian virus 40 large T antigen is required to functionally inactivate RB family proteins. *Mol. Cell. Biol.* **18,** 1408–1415.

zur Hausen, H. (1999). Viruses in human cancers. *Eur. J. Cancer* **35,** 1878–1885.